University of Alberta

The Incorporation of Isomers of Conjugated Linoleic Acid (CLA) into Egg Yolk Lipids, by feeding the Laying Hen.

By

Sean Adrian Rutten Jones ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

In

Nutrition and Metabolism

Department of Agriculture, Food and Nutritional Science

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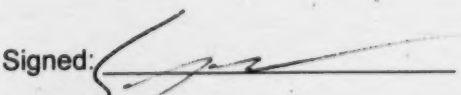
Signed: 

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The undersigned certify that they have read, and recommend to the faculty of Graduate Studies and Research for acceptance, a thesis titled The Incorporation of Physiologically Active Isomers of Conjugated linoleic Acid (CLA) into Egg Yolk Lipids, though Dietary Modifications of the Laying Hen Diet submitted by Sean Adrian Rutten Jones in partial fulfillment of the requirements for the degree of Master of Science in Nutrition and Metabolism.

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For my parents

Who showed me that

anything is possible

in God

ABSTRACT

This study was designed to determine if conjugated linoleic acid (CLA) is incorporated into egg lipids through dietary CLA supplementation. Forty Single Comb White Leghorn layers (28 weeks of age) were randomly assigned into four treatments of varying CLA levels, 0, 0.01, 0.5 and 1g CLA/kg diet. CLA replaced an equivalent amount of linoleic acid in the diet. Eggs were collected over 36 days. Feed consumption and body weight was also monitored. CLA content of the yolk lipids was analyzed by gas liquid chromatography. The groups fed 0.5, and 1.0 g CLA/kg feed had significantly higher levels of CLA present in the egg yolk lipids versus the control and 0.01 g CLA/kg diet groups after 7 days ($P<0.0004$). Incorporation of CLA in egg lipids reached the highest level on days 24, and 36 respectively. CLA enrichment in egg lipids in the 1.0 g CLA/kg diet group was similar to that present in ruminant animal food products at a level of ~3 mg CLA/g fat.

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LIST OF ABBREVIATIONS

AA	arachidonic acid
BF_3-MeOH	boron trifluoride in methanol
CLA	conjugated linoleic acid
DMBA	dimethylbenz(a)anthracene
ddH_20	double deionized water
GLC	gas liquid chromatography
KOH	potassium hydroxide
LA	linoleic acid
PKC	protein kinise C
TPA	12-O-tetradecanoylphorbal-13-acetate

Chapter 1

Introduction and Background

A) Introduction and Rationale

Concern by both the public and health professionals over the possible health risks of consuming high fat diets is prevalent. This concern stems from the link between dietary fat and chronic diseases such as cancer and obesity. It is estimated that in the United States ~35% of deaths due to cancer are related to diet (Doll and Peto. 1981). Epidemiological evidence has shown a relationship between dietary fat consumption and the incidence of colon, prostate, and breast cancer (Belury 1995). Consumption of animal food products, such as beef, pork, and poultry is perceived to be 'unhealthy', due to the fat content and has lead to decreased consumption of these foods from the North American diet over the past 20 years (Robbins 1990; US Department of Commerce 1992)

Certain types of dietary fats may posses anti-carcinogenic properties. These fats include long chain n-3 (Belury 1995), and conjugated isomers of the essential fatty acid, linoleic acid (reviewed by Ip 1994; Belury 1995). Conjugated derivatives of linoleic acid (CLA) have received considerable attention, as it has been found that isomers of this compound have anti-cancer activity (Scimeca et al. 1994), immune enhancing qualities (Cook et al. 1993; Miller et al. 1994), weight reducing effects (Scimeca et al. 1994; Belury et al. 1997a) and possible anti-atherogenic properties (Lee et al. 1994; Nicolosi et al. 1996; Nicolosi et al. 1997). These effects have been observed at levels below 1% of the total energy in the diet (Ha et al. 1989). CLA is predominant in food items produced from

ruminant animals by the rumen microorganism, *Butyrivibrio fibrisolvens* (Ha et al. 1987; Ha et al. 1989; Chin et al. 1992b). CLA is also found in trace amounts in non- ruminant animal products, and vegetable oils (Ha et al. 1989; Ha et al. 1987; Chin et al. 1992b). Currently, the average intake of CLA is estimated to be around several hundred mg per day (Ha et al. 1989; Fritsche et al. 1998a). Based on animal data, it is estimated that a level of approximately 3 g per day of CLA would be required to observe beneficial effects in humans (Ha et al. 1989).

It is possible to change the lipid profiles of food products, such as eggs, through dietary manipulation of the laying hens (Cruickshank 1934). This has been demonstrated in the enhancement of omega-3 fatty acid content in eggs by feeding laying hens diets containing 0.05-3% w/w fish oil (Hargis et al. 1991) or 3% α-linolenic acid from flax seed (Sim et al. 1995). In this respect, it is possible that the development of CLA enriched foods may be able to play vital role in diet based cancer prevention in human populations.

The objective of this literature review is to provide an overview of the chemistry and food sources of CLA, the chemo protective effects of CLA, and the proposed mechanisms of the anti carcinogenic action of CLA. It will also address the weight reduction properties of CLA, and the potential impact that CLA research can have on the poultry industry, and the significance of CLA for human nutrition.

B) Review of literature

1) Chemistry and Food Sources of CLA

Conjugated linoleic acid (CLA) refers to a family of positional and geometric isomers of the essential fatty acid linoleic acid, an 18 carbon unsaturated fatty acid. Linoleic acid has two cis double bonds located on carbons 9 and 12. Comparatively, the two double bonds of CLA are conjugated, exist in either cis or trans geometry and in various positions. Geometrical isomers of 7:9, 8:10, 9:11, 10:12, and 11:13 isomers of 18:2 have been identified (Christie et al. 1998; Kramer et al. 1998). Therefore there may be potentially be as many as 24 isomers of CLA.

Only a few of these isomers occur naturally in foods For the most part, the c9, t11-18:2 isomer is the predominant form found in foods (Figure 1-1), especially in meats, cheese and dairy products, comprising greater than 76% of all of the CLA isomers in these products (Chin et al. 1992b). A sample of CLA concentrations in food is listed in Table 1-1.

Animal studies using synthetic mixtures of CLA suggest that CLA is an effective modulator of carcinogenesis by affecting initiation, promotion, progression, and/or regression (reviewed in (Belury 1995; Ip 1994). The c9,t11-18:2 isomer has been implicated as the biologically active form. This is because it is readily incorporated into the phospholipid membranes of cells (Jiang et al. 1996),and is the predominant isomer in the neutral lipid fraction of cells(Chin et al. 1994b; Ip et al. 1995). However, it is important to recognize that the c9,t11-18:2 isomer is the biologically abundant form of CLA, and the other possible isomers are usually derived synthetically.

In a study by Ha et al, they found that c9,t11-18:2 alone or a mixture of CLA isomers produced the same effects in cancer prevention (Ha et al. 1987). It has also been found that CLA fed as free fatty acids produce the same effects as feeding CLA in the triglyceride form (Ip et al. 1995). This suggests that all isomers may be involved in the chemoprevention observed by CLA ingestion.

2) Cancer protection and cancer modulation by CLA

Several studies show that tumorigenesis is effected by CLA. Using a two-stage mouse skin cancer model, Ha et al (1987) were the first study to conclusively show that CLA had anti-carcinogenic activity was performed by (Ha et al. 1987),. Synthetically prepared CLA was topically applied at 7 days, 3 days and 5

minutes prior to exposure to the carcinogen 7-12-Dimethylbenz[a]anthracene (DMBA) (Ha et al. 1987). At one week after initiation, mice were given topical applications, twice a week, of 12-O-tetradecanoylphorbol-13-acetate (TPA) to effect tumor promotion. The CLA group showed a lower incidence (~15% reduction) and fewer tumors (~ 50%) when compared to control mice that received topical linoleic acid (Ha et al. 1987).

Belury at al. (1996) used the two-stage mouse skin cancer model, to look at tumor promotion specifically. Mice were fed varying CLA free diets during initiation, and then switched to varying levels of CLA (0-1.5% w/w) during tumor promotion with 12-O-tetradecanoylphorbol-13-acetate (TPA). At 22 weeks of TPA treatment, an inverse correlation between tumor yield and level of CLA in the diet was observed. Diets with no CLA averaged 6.2 tumors while diets with 1.5% w/w CLA averaged 4.3 tumors per mouse ($p<0.05$) (Belury et al. 1996).

Ha et al, (1990) found benzo[a]pyrene (BP) induced forestomach tumors in ICR female mice were reduced by oral CLA administration. Feeding CLA (0.1 ml) by gavage 4 and 2 days prior to each weekly oral dose of BP (2 mg) over a 4 week period, reduced the number of neoplasms by ~50% over controls and reduced tumor incidence by 10-21% in three separate experiments (Ha et al. 1990).

Ip et al. (Ip et al. 1991) found similar results when rats were fed AIN-76 diets supplemented with levels of 0, 0.5, 1.0 and 1.5 % (w/w) synthetically prepared

CLA for two weeks prior to oral intubation of DMBA (10 mg). The animals continued their supplemented diets until 24 weeks after carcinogen administration. A dose dependent reduction in tumor number (33-60%), multiplicity (33-60%), and tumor incidence (17-50%) was observed over the control group (Ip et al. 1991).

3) Possible mechanisms for CLA anti-cancer action

There are several proposed mechanisms as to how CLA effects tumorigenesis. Since CLA is incorporated into cell membrane phospholipids (Ip et al. 1991; Ha et al. 1990), several cellular events may be affected. Replacement of other polyunsaturated fatty acids (PUFA's) with CLA may affect oxidative stress, eicosanoid synthesis, and signal transduction (Belury 1995). Another possibility is that CLA may be converted into active metabolites that modulate carcinogenesis (Ip et al. 1997b).

a) Oxidation effects

Studies performed in vivo and in vitro, show CLA to be a potent antioxidant. At 0.25% w/w CLA in the diet or higher, CLA reduced the test scores of a biomarker (TBARS test) used to assess oxidation in biological systems in vivo (Ip et al. 1991; Ha et al. 1990), There is however evidence which casts doubt on the theory that CLA is an anti-oxidant (Scimeca et al. 1994).

An alternate hypothesis is that CLA has pro-oxidant actions in cancer cells. This mechanism is proposed based on the information that PUFA's induce differential cytotoxicity of cancer cells compared with normal transformed cells (Belury 1995). Induction of cytotoxicity by CLA has been shown in several cell lines and in MCF-7 mammary cells. This indicates that CLA is a more potent cytotoxic agent than linoleate or ß-carotene (reviewed by Belury 1995). This has been suggested to be due to the nature of the conjugated double bond system, allowing for more efficient electron trapping. An enhanced likelihood of superoxide anion generation would occur from this, leading to increased cytotoxicity of cancer cells (Belury 1995).

b) Eicosanoid synthesis

CLA may also compete with other PUFA's for incorporation into membrane phospholipids (Ip et al. 1991; Ha et al. 1990; Sugano et al. 1997). Altered eicosanoid production and function could result through displacement of certain PUFA's (reviewed by Belury et al. 1997a). A change in eicosanoid production would influence several processes required for tumorigenesis, such as cell proliferation (induction of ornithine decarboxylase and DNA synthesis) and inflammation (local and systemic immune responses) (Belury 1995).

Arachidonate-derived eicosanoids are known to modulate carcinogenesis. These include prostaglandin E_2 (PGE_2), $PGF2\alpha$ and PGD_2. These and other eicosanoids are produced via the cycloxygenase pathway, and lipoxygenase pathways. Various eicosanoids has been shown to have slightly different modulatory effects on tumorigenesis, although PGE_2 is most often associated with tumor induction. Studies using PGE_2 inhibitors showed a positive correlation with decreased tumorigenesis in the mammary gland (Cunningham et al. 1997; Belury 1995). It has been proposed that CLA may compete with linoleic acid for biosyntheses of arachidonic acid (AA) and the corresponding prostaglandins and leukotrienes(reviewed by Shultz et al. 1992).

Animals consuming diets containing CLA are found to have decreased arachidonic acid content in select tissues, suggest that CLA may alter the products of the cycloxygenase and lipoxygenase pathways (Cook et al. 1993). Other experiments in mice have found that CLA is incorporated into all tissues examined, including the brain (Sugano et al. 1997). Interestingly, CLA decreased the amount of 16:1, 18:1, 18:2 n-6, and total n-6 fatty acids, but increased the >22 carbon n-3 and total saturated fatty acids (Li et al. 1997). In contrast it has been found that AA and LA in the liver, colon (Liew et al. 1995), and mammary gland (Ip et al. 1997a) were not altered by CLA in the diet. Cook (1993) has shown that dietary CLA (.5% w/w) partially displaced AA in rat abdominal fat pads. AA was decreased and CLA content was increased significantly ($p< 0.05$) (Cook et al. 1993). Diets where AA was replaced with other fatty acids, such as

n-3 PUFA's, has been correlated with a reduction in PGE_2, and tumor formation (reviewed by Belury et al. 1997a).

In other studies, mouse keratinocytes incubated with CLA, LA or the tumor promoter AA, then treated with TPA, resulted in significantly less PGE synthesis in CLA incubated cells compared to the other groups (Belury et al. 1997b). Further it was found that CLA decreased ex-vivo PGE_2 production in bone, and they speculate that CLA inhibits delta-9-desaturase (Li et al. 1997). The use of eicosanoid synthesis inhibitors to investigate potential mechanisms of CLA suggests that the effects of CLA were mediated through lipoxygenase inhibition (Cunningham et al. 1997). Interestingly, in the presence of n-3 and n-6 PUFA's, CLA did not exhibit an effect on PGE_2 synthesis ex-vivo, but did exert effects on macrophage cytokine production (Turek et al. 1997). In summary, this evidence supports the hypothesis that CLA modulation of tumorigenesis involves PGE synthesis, and an influence on the cycloxygenase and lipoxygenase pathways.

c) Signal transduction

A third possibility is that CLA elicits an effect through affecting signal transduction. Signal transduction refers to the sending of messages from outside the cell through the plasma membrane to the nucleus. This occurs through the stimulation of the cell membrane, which triggers a cascade of enzymatic and biochemical events that regulate a variety of cellular processes. Of particular

interest is protein kinase C (PKC). It is thought that loss of the regulation of PKC may lead to abnormal cell processes. It has been found that the tumor promoter TPA is a potent activator of PKC, thus CLA down regulation of PKC has been suggested as having a possible role in tumorigenesis (Reviewed by Cunningham et al. 1997; Belury 1995).

Phospholipids are required for activation of PKC, and it has been shown that many species of PUFAs activate PKC at different levels. It has been suggested that free fatty acids activate PKC by partially removing the requirement for a phospholipid cofactor. It has been suggested that CLA may exert an effect on PKC, although to date, CLA has not been shown to activate PKC to the same extent as other PUFAs, and its modulation of PKC is yet to be determined in vivo (reviewed by Belury 1995). The role of CLA in signal transduction may be important in the slowing the development of cancer.

d) Active Metabolites

It has been hypothesized that CLA's action is through the production of active metabolites. Several studies looking at this possibility have determined that products of CLA's metabolism may include furan fatty acids (Yurawecz et al. 1995) as well as C20:3 (▲8,12,14), C20:4 (▲5,8,12,14),and 20:4 (▲5,8,11,13) (Sebedio et al. 1997). It is possible that the furan fatty acids produced could partially explain the observed anti-oxidant properties associated with CLA

(Yurawecz et al. 1995). Other evidence show that CLA may be desaturated and elongated (Belury et al. 1997a; Banni et al. 1995; Sebedio et al. 1997). Thus, elongated conjugated dienes may have important biological effects in competing with other long chain fatty acids in eicosanoid and prostaglandin action (Lui 1997; Park et al. 1997; Sugano et al. 1997; Cunningham et al. 1997). While desaturated and elongated conjugated dienes have been identified (Banni et al. 1995; Sebedio et al. 1997; Belury et al. 1997a), the function of these newly characterized fatty acids is unknown.

4) Other properties associated with dietary CLA

CLA is reported to have other effects not related to cancer, in a wide variety of species, such as anti-atherogenic properties (Lee et al. 1994; Nicolosi et al. 1996; Nicolosi et al. 1997; Gavino et al. 1998), improved immune response (Cook et al. 1993; Miller et al. 1994; Chin et al. 1994a; Hayek et al. 1997; DeVoney et al. 1997), increased feed efficiency and weight gain (Cook et al. 1993; Chin et al. 1994a), and body fat reduction (Pariza et al. 1997). Feeding CLA has been reported to reduce body fat deposits of mice and chickens (Cook et al. 1993; Belury et al. 1997a; Park et al. 1997) by 50% or more (Pariza et al. 1996).

A proposed mechanism for the reduction in body fat and the increase in lean body mass is that CLA significantly reduces heparin-releasable lipoprotein lipase activity resulting in decreased triacylglyceride and glycerol concentrations in fully differentiated 3T3-L1 adipocytes (Pariza et al. 1997; Park et al. 1997). Epididymal adipocytes from rats fed 0.5% w/w CLA showed increased norepinephrene-induced lipolysis and hormone sensitive lipase activity. Thus the effect of CLA on body composition appears to be involved in reducing fat deposition, increasing lipolysis in adipocytes, and possibly enhanced fatty acid oxidation in adipocytes and muscle cells. (Pariza et al. 1997; Park et al. 1997).

5) Importance of CLA to poultry research

The findings related to the consumption of CLA in animals and the possible benefits in humans have several important implications for the poultry industry. Of particular importance is the potential of CLA to improve immune responses in poultry leading to a decrease in catabolism during infection. Several studies by Cook et al. and Miller et al, (Cook et al. 1993; Miller et al. 1994) have shown that feeding CLA at levels of 0.5% CLA (w/w) is sufficient to control catabolic responses due to endotoxin injection in rats and chicks. The reduced catabolic effect translates to little or no decrease in body weight, and an increase in lean body mass following endotoxin injection. There was no indication that the maintenance of lean body tissue following an immune challenge was due to

CLA suppressing immune function. (Cook et al. 1993; Miller et al. 1994). It is possible that the ability of CLA to prevent immune-induced catabolism is related to the modulation of the eicosanoid pathway, in particular PGE_2. CLA supplementation has a possible use to offset the common loss of lean body mass following vaccination (Cook et al. 1993; Miller et al. 1994).

CLA has also been shown to be effective as a growth factor, and shown to improve feed efficiency (Chin et al. 1994a; Miller et al. 1994; Pariza et al. 1997). Some trials with broiler hens have shown a decrease of 10% in feed consumption for every kg body weight gain (Cook 1995). Since broilers consume ~19 billion kg of feed a year in the U.S., a 2.5% decrease could translate into a savings for the U.S. broiler industry of 544 million kg of feed or 108 million dollars US (Cook 1995). This could also decrease the environmental impact as less feed means less manure production (Cook 1995).

The ability of CLA to reduce body fat, increase lean body mass, and increase feed efficiency, has important implications for the pathological condition, common to many laying hens, fatty liver syndrome. Fatty liver syndrome is caused either by the excess caloric intake of the hens, or by a deficiency in methionine or choline intake (Rothenbacher et al. 1972; Squires and Leeson 1988). The effects of this condition are a result of the hepatocytes becoming distended with fat vacuoles, resulting in an enlarged fatty discolored liver that may rupture causing death (Rothenbacher et al. 1972; Squires and Leeson

1988; Branton et al 1995). The use of CLA as a means to reduce fat deposition may be a method to control or reverse this disease.

Another role that CLA can play in the poultry industry is in the development of novel food products. Products, such as the omega-3 enriched eggs, have been developed for consumers who desire another source of n-3 fatty acids in their diet, and the health benefits associated with n-3 PUFA's. This is achieved through dietary modification of the laying hens diet (Hargis et al. 1991)

It has long been known that the fatty acid composition of egg yolk is readily altered by dietary manipulation, while total lipid content remains the same (Cruickshank 1934). The incorporation of specific fatty acids, or lipid soluble vitamins into eggs is an area of research receiving a lot of attention. Currently there is a growing movement towards non-pharmacological cancer preventative alternatives and foods (Hargis et al. 1991; Jiang et al. 1993).

The possibility exists to develop a feeding regime to produce CLA rich eggs. The development of a CLA rich egg, and successful marketing, may benefit the egg producer and consumers who desire a nutrition based route for cancer prevention.

6) Importance to human and scientific knowledge

Although clinical studies with CLA have yet to be reported, this molecule has tremendous potential in a number of areas, the most intriguing being its anti-cancer properties. Currently, the recommendations to lower the risk of cancer include a reduction in total fat to less than or equal to 30% total energy, and an increase in consumption of fruits, vegetables and grains. Especially foods that are low in fat, high in fiber, and high in anti-oxidants and phytochemicals. It is also advised to avoid foods containing carcinogens and mutagens (Belury 1995). Animal products, such as meat, dairy, and poultry products, are often associated with high levels of mutagens and carcinogens, but they also contain CLA. (Ha et al. 1987;Ha et al. 1989; Chin et al. 1992a) In the typical diet, it is estimated that a few hundred milligrams of CLA are consumed per day (Ha et al. 1989; Fritsche et al. 1998b). Although extrapolation from animal studies to humans is complex, a direct extrapolation for humans, based on a 70 kg man, is approximately 3 grams of CLA per day to reach a level similar to the 0.1% CLA (w/w) in the diet, the level where CLA shows an effect in animal models (Ha et al. 1989).

While present consumption of CLA is much lower than the levels suggested to see an effect, it has been shown that dietary sources of CLA can increase the levels of CLA found in human plasma and tissue. Huang et al (1994), has shown that supplementing cheddar cheese to ovo-vegetarian men, at a level of 112g/day (providing 178.5 mg of CLA/day) increased plasma CLA:LA molar

ratios up to 30% compared to the control group (Huang et al. 1994). This study shows that even small increases in CLA consumption will increase CLA in humans. It has also been shown that some monogastric animals are capable of endogenous production of CLA, from either carbon centered free radical oxidation of linoleic acid (Dormandy et al. 1987) and/or from bacterial microflora (Harrison et al. 1985). Indirect evidence regarding human consumption of CLA shows it to be a beneficial component in the diet. In a large scale, a longitudinal study involving 4697 women, initially cancer free, Knekt et al. (1996) suggests that women consuming higher levels of dairy products have a decreased incidence of breast cancer. However, there still remains much work to gather specific epidemiological data relating CLA intake to the efficacy of CLA.

These findings justify the need for food technology advancements to increase the level of CLA in food products. This could facilitate the achievement of CLA in the diet closer to the level of 3 grams per day.

7) Summary and Objectives of this study

In conclusion, CLA appears to play many different roles that may impact human health and nutrition. Not only does CLA appear to have a place as a chemoprevention agent, but also it has been linked to improved immune function, weight control, and atherogenesis. This molecule also appears to

influence a variety of mechanisms , and act on many different species. The potential implications of CLA for poultry and egg production are interesting, and warrant investigation.

Influences in body weight, increased immune function, and the development of novel food products, are intriguing prospects for poultry research. The objective of this study is to determine the short term and long term effects of CLA supplementation on the CLA content in eggs, CLA distribution in the body fat of the laying hens, and on the weight changes of the Shaver 2000 laying hen.
It is hypothesized that supplementing CLA at four different levels (0, 0.01, 0.5, 1.0 g of CLA/kg feed) will produce short term and long term effects on layer performance. It is specifically hypothesized that dietary CLA will:

- incorporate into the egg yolk fraction in a dose related fashion
- reduce weight gain in the laying hens in a dose dependent manner
- reduce fat accumulation in the liver of the laying hen
- improve feed efficiency in a dose dependent manner

8) Thesis Organization

A brief introduction of this area and a relevant literature review is presented in chapter 1. The results along with a brief introduction and discussion are presented in chapter 2. Chapter 2 will be submitted the Journal of Nutrition for publication. A general discussion along with possible extension of this work is presented in chapter 3.

Figure 1-1 Structure of the c9,t11 Isomer of CLA

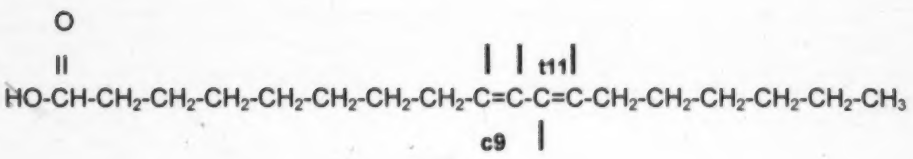

Table 1-1 REPRESENTATIVE CONCENTRATIONS OF CLA IN FOODS
(Chin et al. 1992b)

Food	Total CLA (mg/g fat)	c9,t11 isomer%
Meat		
Fresh ground beef	4.3	85
Veal	2.7	84
Chicken	0.9	82
Turkey	2.5	76
Egg yolk	0.6	71
Seafood		
Salmon	0.3	n/a
Shrimp	0.6	n/a
Cheese		
Romano	2.9	92
Cheddar	3.6	90
Dairy		
Homo Milk	5.5	92
Butter	4.7	88
Vegetable oil		
Safflower	0.7	44
Olive	0.2	47

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Chapter 2

The Incorporation of Isomers of Conjugated linoleic Acid (CLA) into Egg Yolk Lipids, by feeding the Laying Hen.

A) Introduction

Both public and health professionals have concern over the possible health risks of consuming high fat diets. The link between dietary fat and chronic diseases such as cancer, obesity and is at the head of these concerns. It is estimated that in the United States ~35% of deaths due to cancer are related to diet (Doll et al. 1981). Epidemiological evidence has shown a relationship between dietary fat consumption and the incidence of colon, prostate, and breast cancer (American Institute for Cancer research.1997 Consumption of animal food products, such as beef, pork, and poultry is perceived to be 'unhealthy', due to the fat content, thus contributing to decreased consumption of these foods from the North American diet over the past 20 years (Robbins 1990; US Department of Commerce 1992).

Certain types of dietary fats may posses anti-carcinogenic properties. These fats include long chain n-3 fatty acids (Belury 1995), and conjugated isomers of the essential fatty acid, linoleic acid (reviewed in (Ip 1994; Belury 1995). Conjugated derivatives of linoleic acid (CLA) have received considerable attention, as it has been found that isomers of this compound have anti-cancer activity (Scimeca et al. 1994) immune enhancing qualities (Cook et al. 1993; Miller et al. 1994), weight reducing effects (Scimeca et al. 1994; Belury et al. 1997) and possible anti-atherogenic properties (Lee et al. 1994; Nicolosi et al. 1996; Nicolosi et al. 1997). These effects have been observed at levels below 1% of the total energy

in the diet (Ha et al. 1989). CLA is predominantly in food items produced from ruminant animals by the rumen microorganism, *Butyrivibrio fibrisolvens* (Ha et al. 1989; Ha et al. 1987; Chin et al. 1992). CLA is also found in trace amounts in non- ruminant animal products, and vegetable oils (Ha et al. 1989; Ha et al. 1987; Chin et al. 1992). Currently, in North America, the average intake of CLA is several hundred milligrams per day (Ha et al. 1989; Fritsche et al. 1998a). It is estimated that a level of approximately 3 g per day of CLA would be required to observe beneficial effects in humans (Ha et al. 1989).

It is possible to change the lipid profiles of food products, such as eggs, through dietary manipulation of the laying hens (Cruickshank 1934). This has been demonstrated in the enhancement of n-3 fatty acid content in eggs (Hargis et al. 1991; Sim et al. 1995). In this respect, it is possible that the development of CLA enriched foods may be able to play vital role in diet based cancer prevention in human populations.

CLA appears to play many different roles that may impact human health and nutrition. The development of novel food products to influence body weight, and enhanced immune function, are intriguing prospects for the poultry industry. In the present study, Shaver 2000 Single Comb White Leghorn hens were fed diets containing various levels of CLA to determine the short term effects of CLA supplementation on the CLA content of eggs, and the long term effects on body

composition and weight changes of the Shaver 2000 laying hen. Feed intake was also among the variables studied.

B) Materials and Methods

Stocks and Diets. Forty Shaver 2000 Single Comb White Leghorn pullets were reared in floor pens to 18 weeks of age. The rations were provided as follows: Chick starter from 0 to 6 wk; Grower ration 1 from 6 to 16 wk; and Layer ration from 16 to 29 wk. At 18 wk, hens were moved to individual laying cages. At 29 wk, hens were fed a modified Layer ration until end of lay. The four diets consisted of a control diet (standard layer ration), a low CLA diet containing 0.01g CLA/kg diet (0.001% w/w, 0.04 % total added fat), a medium diet with 0.5 g CLA/kg feed (0.05% w/w, 1.8% total added fat), and a high CLA diet having 1.0 g CLA/kg feed (0.1% w/w, 3.7 % added fat). The nutrient analysis of each ration is listed in Table 2-1. Feed was provided *ad libitum* from individual feed containers, while clean drinking water was available at all times.

Management and Experimental Design. Pullets were reared in light tight floor pens with a bird density of ½ sq. ft per bird until 10 wk, then 2 sq. ft per bird until 18 wk. Chicks were subjected to a photo schedule of 23 hours light (L) to 1 hour dark (D) (23L:1D), which was reduced to 8L:16D at 4 d and maintained until 18 wk of age. Beak trimming was performed between 5-8 days of age. At 18 wk of age, pullets were moved to individual laying cages and fed a standard layer

diet. Once in laying cages, the photo schedule was increased to 11L:13D, and was increased by ½ hr of light until it reached 14L:10d. At 29 wk, 40 pullets were weighed, and assigned into one of four treatment groups, a control group (standard layer diet), or CLA enriched diets of 0.01 g CLA/kg diet, 0.5 g CLA/kg diet, or 1.0 g CLA /kg diet. Pullets were maintained on these diets for 36 days and eggs were collected on days 1-12, 24 and 36. Individual feed intakes were calculated weekly. Each bird was provided with an individual feed container that was inaccessible from neighboring birds. Intakes were calculated by feed weigh-back, through recording the feed given, and subtracting the initial starting weight, and the end weight to give the feed consumed. Body weight was measured bi-weekly. After 36 days, six hens from each group of ten were returned to the general laying population, and the remaining hens from each group were fed their respective diets until they reached the age of 68 weeks. Feed intakes and body weight measures were taken every four weeks until end of lay.

All birds survived until 68 wk of age. On the afternoon prior to being sacrificed, the birds were subjected to feed withdrawal over night (12-20 h) to permit gut content clearance. All experimental procedures performed on live birds were within the principals and guidelines approved by the University of Alberta, Faculty of Agriculture, Forestry, and Home Economics, Animal Policy and Welfare Committee.

Synthesis of CLA. CLA was obtained from Ma et al. (Ma 1998). It was produced from linoleic acid purified from safflower oil, and isomerized using a

modified method described by Chin et al. (1992). The CLA obtained had a purity of 95%. The majority of the isomers present were in the 9c, 11*t* –18:2 and the 10t,12c isomer which account for 94.6% of the total CLA content (Ma 1998) (Figure 2-1)

Preparation of Experimental Diets. Standard Layer rations (University of Alberta Edmonton Research Station) were prepared with the exclusion of canola oil from the mixture. Canola oil (Country Harvest 100% Pure Canola oil, Lucerne Foods LTD, Vancouver, B.C.) was purchased from commercial sources.

Diets were prepared individually in quantities of 35 kg, estimated to last ~30 days, and diets were always prepared in a consistent order starting with the control diet, 0.01g CLA/kg feed, 0.5g CLA/kg feed, and 1.0g CLA/kg feed to avoid CLA contamination in the lower CLA concentration feeds.

Addition of CLA into canola oil fraction - The amount of canola oil that was required for a standard layer ration (control diet) of 35 kg of feed is 959 g (27.4% w/w). The amount of CLA required for each concentration was calculated (factoring in the 95% purity of CLA). A 1.5L beaker was zeroed on a digital weigh scale and the CLA was added to the beaker. Canola oil was then added to the beaker until the desired weight of 959g was achieved. The last 5 g of canola oil was added with a pipette to ensure a weight of 959 g ± 0.5 g. The oil was stirred for 10 minutes with a large magnetic stirrer to ensure uniform.

a. Preparation of pre-mix – The pre-mix consisted of adding the canola oil (959 g) to 6.041 kg of canola-free standard diet, for a total of 7 kg of pre-mix. Approx. 2/3 of the canola-free diet was added to a 20 Kg Hobart industrial mixer (Hobart, Troy, Ohio). The oil was then added to the feed and mixed for 3 min. The remainder of the dry diet was added to the beaker and hand mixed to remove the remaining oil from the beaker walls and the magnetic stirrer, it then was added to the industrial mixer and blended for 10 min. A 5 L pail was then zeroed on a digital weigh scale, and the pre-mix was then added and weighed to ensure the complete transfer of 7kg total (+/- ~12 g). The pail was covered and stored at 5°C until mixed into the full diet.

b. Preparation of Diet - The remaining 28 kg of feed was weighed out into 40L plastic feed drums on a 100 kg livestock scale. Approx. ¾ of the dry feed was added to a (100 kg) cone feed mixer along with the pre-mix. The remaining dry feed was used to remove any of the remaining oil from the pre-mix pail. The diet was mixed for ~12 min to ensure uniformity. The feed was then returned to the 40L plastic drums, lids were added, and the feed was stored at 4°C.

Egg production. Individual daily egg records were kept for the hens until 68 wk. The incidence of abnormal eggs (soft-shell, shell-less, double yolked,

broken, abnormal or pecked) was recorded as an indication of shell quality. Egg production was expressed as average hen-day production, calculated from the total eggs divided by the number of days. Hen-day production was calculated for the entire study. Egg and yolk weights were measured for egg collected on days 1-12, 24 and 36. Yolks were separated form the albumen using a small home egg separator, and the yolks were gently rolled on a paper towel to remove any adhering albumen. Yolk contents were stored in 15 ml scintillation vials at –35°C.

Saponification and Methylation. One g samples of egg yolk were analyzed. Lipid material was extracted by the method of Folch et al. (1957). All lipid extracts were reconstituted to a volume of 10 ml of chloroform, and duplicate aliquots of 1 ml were taken to perform lipid analysis, these were stored at –35°C.

In a screw cap tube, 5 mg of yolk lipid and 25 μg C19:0 of free fatty acid internal standard were saponified in 2 ml of 0.5 M NaOH-MeOH. Samples were heated for 1 hour at 110°C in a sand bath then cooled. Hexane (2 ml) and 14% w/v BF_3-MeOH (2 ml) was then added to each sample and were methylated at room temperature as described by Werner et al. (Werner et al. 1992). Samples were methylated for 30 min with shaking, and immediately after double distilled water (ddH_2O) (1 ml) was added, samples were then vortexed, and centrifuged at 1000 rpm for 10 min. The upper hexane phase was collected and analyzed by GLC. Fat content was expressed relative to dry lipid weights.

Gas Liquid Chromatographic (GLC) Analysis of CLA isomers in yolk lipids – All GLC analysis was carried out with a Varian 6000 gas chromatograph utilizing a Varian Star Chromatography Workstation (version 4.0). CLA content was quantified using a SP-2560 fused silica capillary column (100 m x 0.25 mm i.d., 0.2 μm film thickness; Supelco Inc, Bellefonte, PA). Samples were dissolved in 300 μl of hexane. The injector port was operated at 250°C. The detector temperature was 270°C. The carrier gas was helium with 2 ml/min flow rate. The column pressure was 50 psi. A 100:1 split mode was used. Samples were eluted off the column using a temperature program set from 130°C to 225°C over 110 min. Samples were injected in duplicate from each lipid extraction.

Carcass Examination. Each pullet was killed by cervical dislocation and the intact carcass was weighed. The weights of the abdominal fat pad, breast muscle (*pectoralis major* and *minor*), liver, oviduct and ovary were recorded. Carcass component weights were expressed as absolute organ weight and as a % of the body weight at processing. Birds were subjectively inspected for incidence of complications, such as internal laying, determined by the presence of yolk material in the body cavity, and for fatty livers, defined by the observance of discolored, large fatty abnormal livers.

All carcass components were labeled and stored together at –20°C. Individual whole body composition was determined for each bird following the procedure

described by Yu et al. (1990). Each carcass was autoclaved for 3.5 h and homogenized using an industrial pressure-cooker and blender. A 250 ml sub-sample of each homogenized carcass was taken in duplicate, and labeled *A* and *B*. All *A* sub-samples were freeze-dried and subjected to proximate analysis in duplicate for dry matter, ash, crude protein, and petroleum ether-extractable lipid content (AOAC 1980). Total lipid content was determined by petroleum ether extraction. For each tissue analysis, a 2% difference between duplicates was accepted, with the exception of a 5% difference allowable for carcass ash content duplicates. Crude protein was analyzed using a LECO Nitrogen Analyzer (LECO, St. Joseph, Michigan)

Statistical Analysis. Fatty acid profiles were derived from GLC analysis using values obtained using c19:0 internal standard as a reference. CLA was quantitatively expressed based on the mean ± SEM from multiple trials.

A two-way ANOVA was used to evaluate CLA content between treatment group and days, as well as the differences in body weights, egg and yolk weights, feed intake, and body composition analysis. Orthogonal comparisons were used to determine significance ($P<0.05$). All analysis was done using SAS® version 6.11. (SAS institute, 1996)

C) Results

1) Phase 1 - Transfer of CLA into eggs in the first 36 days of feeding

Egg Weights, Yolk Weights and Egg Production. No significant differences were observed in egg or yolk weights between levels. There were differences in yolk weights, with day 36 being significantly heavier than all previous days (16.59 g ± 0.35) ($p<0.0001$), and day 24 weights being increased above those of days 1-11 (15.91 g ± 0.39) ($p<0.02$). (Table 2-2). Egg production, expressed relative to hen-day production, was significantly influenced by diet ($p<0.05$), with the control and low groups having the highest laying rates throughout the study, followed by the medium group, then the high CLA fed group (95.26%, 94.05%, 91.62 and 88.84% respectively). In terms of Grade A egg production, it was found that the only change between total and Grade A eggs was in the low group, which significantly dropped in production efficiency as shown in Table 2-3.

CLA Incorporation into Yolk Lipids. Samples were grouped by the level of CLA in the diet (0, 0.01, 0.5, or 1.0 g CLA/kg feed), and by the day the eggs were collected. To assess the relative and absolute abundance of CLA within this study and to make comparisons to other studies, levels were expressed relative to fat content (mg/g fat) (Figure 2-2). The CLA level for all groups at day zero, prior to switching diets, was 0.468 mg CLA/g lipid ±0.058. After 36 days, all

three of the CLA fed groups became statistically higher in their CLA contents versus the control diet and day zero values. Within one week of feeding, the medium (0.5g CLA/kg feed) and the high (1.0g CLA/kg feed) groups showed a 68-73% increase in CLA content compared to their day 0 values ($P<0.0004$). The medium and high groups started showing significant differences between days 9 and 12 day of feeding the respective diets, with the high CLA group being significantly higher ($P<0.0001$) than the medium CLA group. For the medium CLA group, day 24 was significantly higher than day 12 ($P<0.0001$), but showed no significant difference from day 36. The high CLA fed group was significantly increased between days 12 and 24 ($P<0.01$), and again between day 24 and 36 ($P<0.003$). The high CLA fed group was also significantly higher at days 12, 24 and 36 than the other three groups ($P<0.0001$). The control and low CLA groups were statistically similar between days 0 and 36, although group fed a low CLA diet showed a ~50% increase in CLA content over its day zero value by day 36 ($P<0.007$). The low and control data points for days 7-12 were assumed not to be different between day 0 and day 24, thus they were not investigated as of yet. In terms of the individual isomer incorporation after 36 days, the c9,t11 isomers were preferentially incorporated into the yolk lipids over the t10,c12 isomers ($p<0.0001$) in all groups, showing a ratio of 4:1 in the high group and a ratio of 30:1 in the medium group (Figure 2-3).

2) Phase 2 – Body Composition changes and feed consumption after long term feeding

Body Weight Changes. There were no significant differences in body weight between all levels of CLA intake until week 5, where the control and low CLA groups were found to be significantly higher than the medium CLA group for the remainder of the study ($P<0.05$), and again at 24 weeks, where the low and control CLA groups became significantly heavier than the high CLA group ($P<0.03$). The weights of the medium and high CLA fed groups did not significantly change over the 28 weeks, while weights of the control and the low CLA groups increased significantly after 24 weeks of feeding (55 weeks of age) ($P<0.04$ and $P<0.001$ respectively) illustrated in Figure 2-4. Comparing body weights through out the study, the control and low CLA group showed no significant difference from each other (1.81 ± 0.025, 1.82 ± 0.042, respectively), but both groups weighed significantly more than the medium and high CLA fed groups (1.64 ± 0.013, 1.72 ± 0.019, respectively) ($P<0.035$), and the medium group was significantly smaller than all groups ($P<0.0007$) shown in Figure 2-5.

Feed Intake. Feed intakes were recorded weekly. Feed intake was expressed in relation to body weight (g feed/kg body weight). Feed Intakes fluctuated from week to week and were statistically different ($P<0.025$) comparing week to week. No differences were found between groups within a given week, except in week five, where the medium CLA fed group (0.05 g

CLA/kg) consumed significantly more ($P<0.008$) than all other groups (Figure 2-6).

The amount of food consumed by each group over the course of the study, relative to body weight, showed that the high and low CLA fed groups ate approximately 4-7% less ($P<0.025$) than the control and medium CLA groups.

Body Composition Analysis. When the animals reached 68 weeks of age, they were sacrificed and an assessment of the liver, ovary, breast muscle, oviduct and fat pad weights were recorded. There were no significant differences between treatment groups for the weights of the liver, ovary, breast, and oviduct measures relative to body weight. Fat pad measures of the low CLA fed group was significantly higher than the other groups ($P<0.009$), while the other groups were statistically similar (Figure. 2-7).

In terms of direct measures of body composition, the high CLA fed group had a significantly lower percent body fat than the medium and low CLA fed groups, while surprisingly this group was not significantly different from the control group. The high CLA group also had significantly higher protein than the low CLA group, but was not significantly different from the control and medium CLA fed groups (Table 2-4).

Observations on the appearance of fatty livers. While not statistically significant, the presence of fatty livers were visually determined in the control and low CLA fed groups only, at a rate of 1:4 or 25%.

D) Discussion

Egg and yolk weights and egg grade were looked at as indicators of adverse effects on egg production or quality. The observation that on days 24 and 36 the egg and yolk weights were significantly heavier than the previous days for all groups, is in accord with data showing that egg and yolk weights significantly increase in weight as the birds age (Thorsteinson et al1997). From the egg production data, we observed a decrease in the production rates of the high and medium CLA fed groups, thus it appears that CLA exerts and effect on the reproduction efficiency of the hens, as compared to the control and low fed CLA groups. The reason for this finding has yet to be determined, and requires further investigation.

The development of CLA enriched eggs may have similar consumer appeal as omega-3 enriched eggs. Products, such as the omega-3 enriched eggs, have been developed for consumers who desire to increase the n-3 fatty acid content in their diet, and the health benefits associated with n-3 PUFA's (Sim et al. 1995). This is achieved through lipid modification of the laying hens diet (Cruickshank 1934; Hargis et al. 1991). It has long been known that the fatty

acid composition of egg yolk is readily altered by dietary manipulation, while total lipid content remains the same (Cruickshank 1934; Hargis et al. 1991).

We found that egg CLA content is increased through modification of the diet. After 36 days, all three CLA fed groups had significantly increased egg CLA content in the eggs compared to the control diet and day zero values. Changes in the medium and high groups (0.5 g CLA/kg, 1.0 g CLA/kg) were observed within one week of feeding. Similar incorporation rates of dietary lipids into yolk lipids have been observed hens fed n-3 fatty acids illustrated in Figure 2-7 (Sim et al. 1995). After 36 days, the eggs from the group consuming the high diet contained the most CLA, being significantly higher than all its previous values ($P<0.003$). The eggs from the medium group reached maximum CLA incorporation after 24 days. On days 24 and 36, the CLA content of the high group was significantly above ($P<0.0001$) that of the medium group on the respective days. The high group did not appear to reach maximum incorporation after 36 days, thus we can assume that it is possible for even higher incorporation of CLA after 36 days. Further study will have to be done to determine the length of time for maximum CLA incorporation when fed a level of 1.0g CLA/kg feed. The amount of CLA that was incorporated after 36 days on the high diet was 3.33 mg CLA/g fat, an amount similar found in ruminant animal products. This amount translates to ~ 15 mg CLA per egg, thus giving an amount of CLA similar to a glass of milk (Chin et al. 1992). It is also interesting to note that the c9,t11 isomeric form of CLA was preferentially incorporated over the

t10,c12 isomer despite being fed in approximately the same ratio in the diet. This in accord to other data showing that the c9,t11 isomer is predominantly incorporated over the t10,c12 isomer when fed to rats in approximately the same ratios (Sugano et al.1997, Winchell et al.1998), furthering the thought that the c9,t11 isomer may be the physiologically form of CLA (Ip 1991).

The incorporation of specific fatty acids, or lipid soluble vitamins into eggs is an area of research receiving a lot of attention. Currently there is a growing movement towards non-pharmacological chemopreventative alternatives and foods (Hargis et al. 1991; Jiang et al. 1993). These results show potential for the development and successful marketing of a CLA rich egg, which would benefit the egg producer and consumers who desire a nutrition based route for cancer prevention.

Feeding CLA has been reported to reduce body fat deposits of mice and chickens (Cook et al. 1993; Belury et al. 1997), as much as 50% or more (Pariza et al. 1996). In this study, we monitored body weight changes and found that the body weights of the medium and high CLA fed groups did not significantly change over the 33 weeks. While the control and low CLA fed groups weight significantly increased after 27 weeks of feeding, and became statistically heavier than the medium CLA fed group after 5 weeks. An interesting observation is that the higher CLA fed groups (0.5 g CLA/kg and 1.0 g CLA/kg) did not become significantly heavier as is usually the case in laying hens

(Squires and Leeson 1988; Branton et al 1995) The incidence of obesity over time is a common and costly problem leading to complications such as fatty livers (Rothenbacher et al. 1972; Squires and Leeson 1988; Branton et al 1995), and although not significant, only the low and control groups developed fatty livers (25%,n=4).

CLA has also been shown to improve feed efficiency (Cook et al. 1993; Chin et al. 1994). In our study, we observed that over the course of the whole study, the high group showed an 8.6% decrease in feed consumption on a feed/kg body weight basis versus the control diet. However, the low group had a 6% decrease, while the medium group had no decrease from the control group. While it is difficult to explain the results of the low group, it may be due to the fact that animals in the low group had a very high % body fat versus the control, medium and high groups (28.5%, 20.7%, 21.9%, and 17.7% respectively). This may lead to a decrease in feed intake in animals that have less metabolically active tissue due to the high fat content, therefore needing less food for nourishment.

E) Conclusions

Overall, CLA was successfully incorporated into egg yolk lipids through dietary modification. It appears possible to attain CLA levels in eggs similar to the CLA levels in ruminant animal products on a per gram fat or per egg basis. The development of novel food products is a growing area of industry, and in the delivery of a nutrition based route for disease prevention, specifically cancer, as

well as, atherogenesis, and possibly obesity, for both animals and humans. It has been extrapolated from animal data, that humans would need to ingest approximately 3 g of CLA/day to see these health benefits (Ha et al 1997). Current estimates of CLA intake in humans are only a few hundred milligrams per day (Fritsche et al. 1998b; Ha et al. 1989). Thus engineering foods having enriched CLA levels brings us closer to this 3 g target. However, CLA supplementation to laying hens may have other benefits other than CLA enriched egg yolks. No adverse effects on egg and yolk weight were observed. Similar trends to those reported in the literature pertaining to increased feed efficiency, maintenance of a stable body weight, and a reduction in fat deposition in the group fed a high CLA containing diet (1.0g CLA/kg feed) were observed. These observations show potential for CLA to be an effective feed additive in the production of eggs.

Table 2-1 Diet Schedule and Nutrient Analysis

Nutrients	Starter 0-6 wk	Grower 1 6-16 wk	Layer 16-29
Crude Protein (%)	21	17	19
ME (kcal/kg)	2900	2800	2800
Linoleic Acid (%)	1.20	1.00	1.40
Methionine (%)	0.42	0.36	0.40
Lysine (%)	1.00	0.75	0.84
Calcium (%)	0.90	0.95	3.80

Table 2-2 Yolk Weight Changes over 36 Days

Days	Yolk Weights
0	15.29 ± 0.30 [a]
1	15.26 ± 0.39 [a]
2	14.57 ± 0.34 [b]
3	14.57 ± 0.32 [b]
4	14.74 ± 0.30 [b]
5	14.97 ± 0.34 [ab]
6	15.03 ± 0.32 [ab]
7	14.99 ± 0.32 [ab]
8	14.79 ± 0.40 [ab]
9	14.97 ± 0.34 [ab]
10	14.80 ± 0.34 [ab]
11	15.14 ± 0.33 [ab]
12	15.49 ± 0.34 [bc]
24	15.90 ± 0.39 [c]
36	16.59 ± 0.35 [d]

[a,b,c,d] Means with no common superscript differ significantly ($P<0.05$)

Table 2-3 Comparisons of Egg Production

Total Egg Production Over Course of Study		Grade A Egg Production Over Course of Study	
Level	Average Hen-Day Production (%)	Level	Average Hen-Day Production (%)
Control	95.26± 1.10 [a]	Control	95.21± 1.10 [c]
Low	94.05 ± 1.10 [a]	Low	90.49 ± 1.10 [ab *]
Medium	91.62 ± 1.10 [b]	Medium	91.53 ± 1.10 [a]
High	88.84± 1.10 [c]	High	88.03± 1.10 [b]

[a,b,c,] Means within a column with no common superscript differ significantly ($P<0.05$)

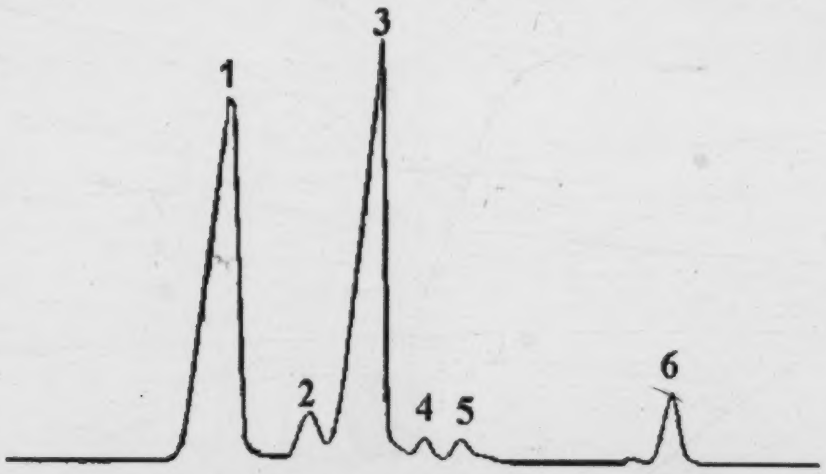
* indicates a change between total and grade A egg production efficiency

Table 2-4 Direct Measures of Body Composition

% Body wt	Lipid	H_2O	Protein
Control (n=4)	20.7±2.48 [ab]	55.1±2.95 [a]	19.8±4.25 [ab]
Low (n=3)	28.5±3.75[c]	48.9±3.15 [b]	18.4±3.75[a]
Med. (n=4)	21.9±2.67[b]	54.2±2.26 [a]	19.7±3.59 [ab]
High (n=3)	17.7±2.62 [a]	57.1±4.05 [c]	20.9±4.97 [b]

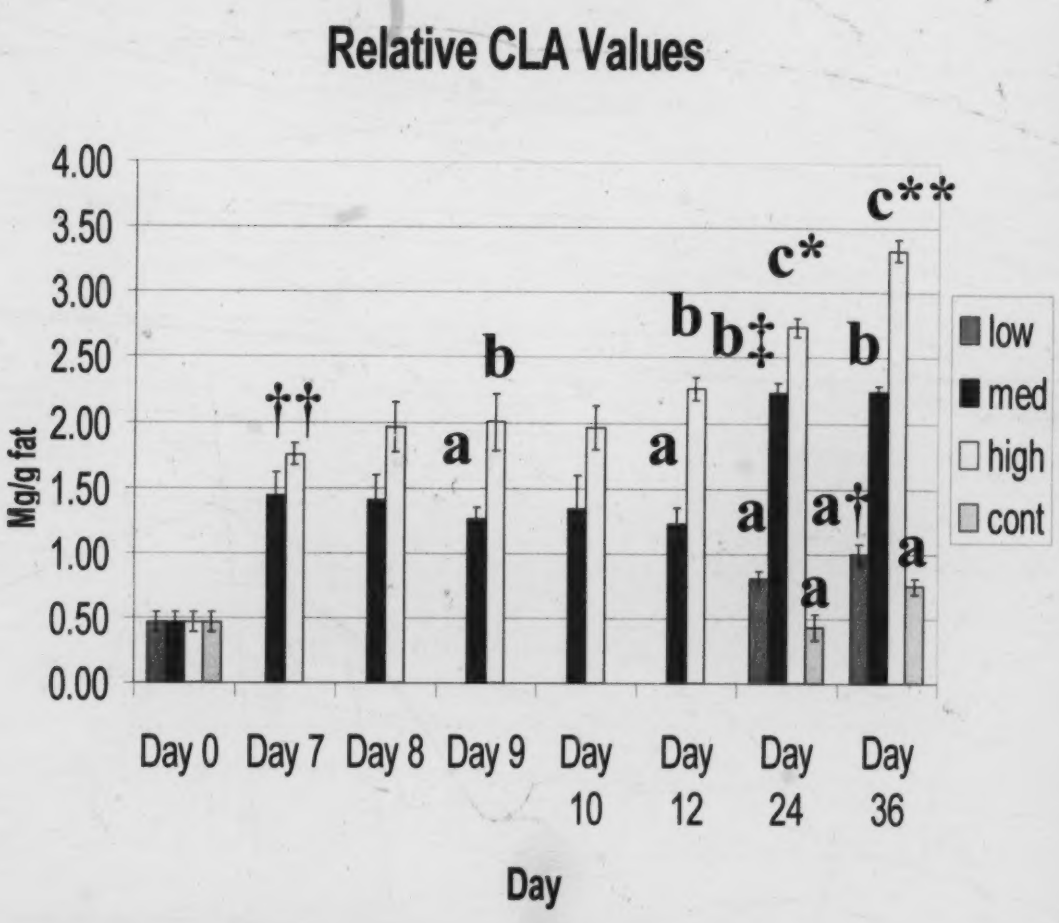
[a,b,c,] Means within a column with no common superscript differ significantly ($P<0.05$)

Figure 2-1 GLC Expansion of Dietary CLA Peaks



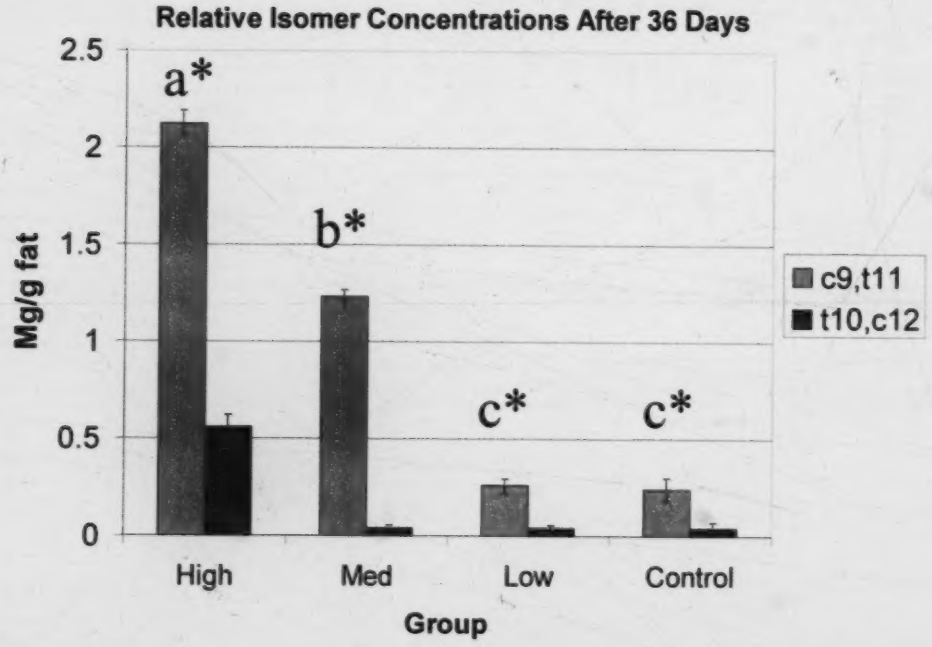
Gas chromatographic expansions of CLA isomers from a SP-2560 fused silica GLC column. CLA produced from LA derived from safflower was separated into 6 peaks. As a percentage of the total mixture, peaks 1,2,3,4,5, and 6 correspond to 45.0±0.7, 3.1±0.7, 46.1±1.0, 0.9±0.04, 1.4±0.1,and 3.5±0.7%, respectively (n=4). Peaks 1,3,and, 6 corresponding to Δ9*c,11*t-, Δ10*t*,12*c*-, and Δ9*t*,11-2-18:2, respectively, were identified in the safflower derived mixture.

Figure 2-2 CLA Incorporation into Yolk Lipids



† Indicates a significant increase from the Day 0 values (P<0.0001)
‡ Indicates the next break point for a significant increase from Days 7-12 for the medium group (P<0.0001)
* Indicates the break point for a significant increase from Days 7-12 for the high group (P<0.01)
** Indicates the break point for a significant increase from all other values (P<0.003)
a,b,c Indicates significant differences within a given day (P<0.05)
The data points for the low and control groups are assumed to similar to statistically similar to days 0 and 24 and were not reported

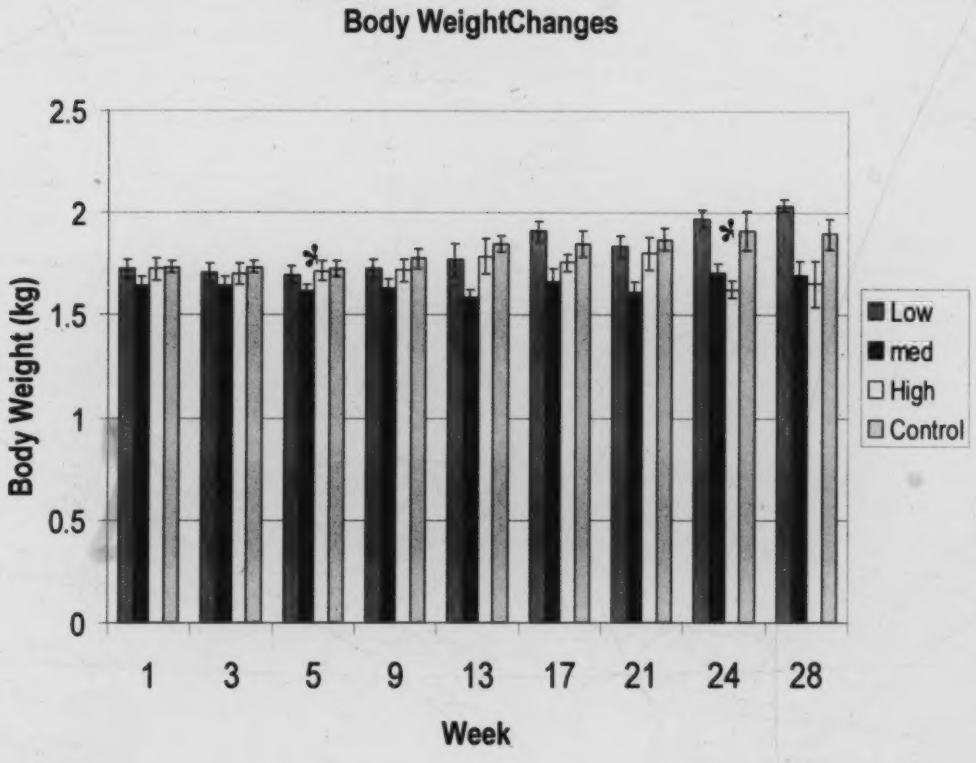
Figure 2-3 CLA Isomer Incorporation into Yolk Lipids



[a,b,c] above a column indicates a significant difference in c9,t11 between groups ($p<0.05$)

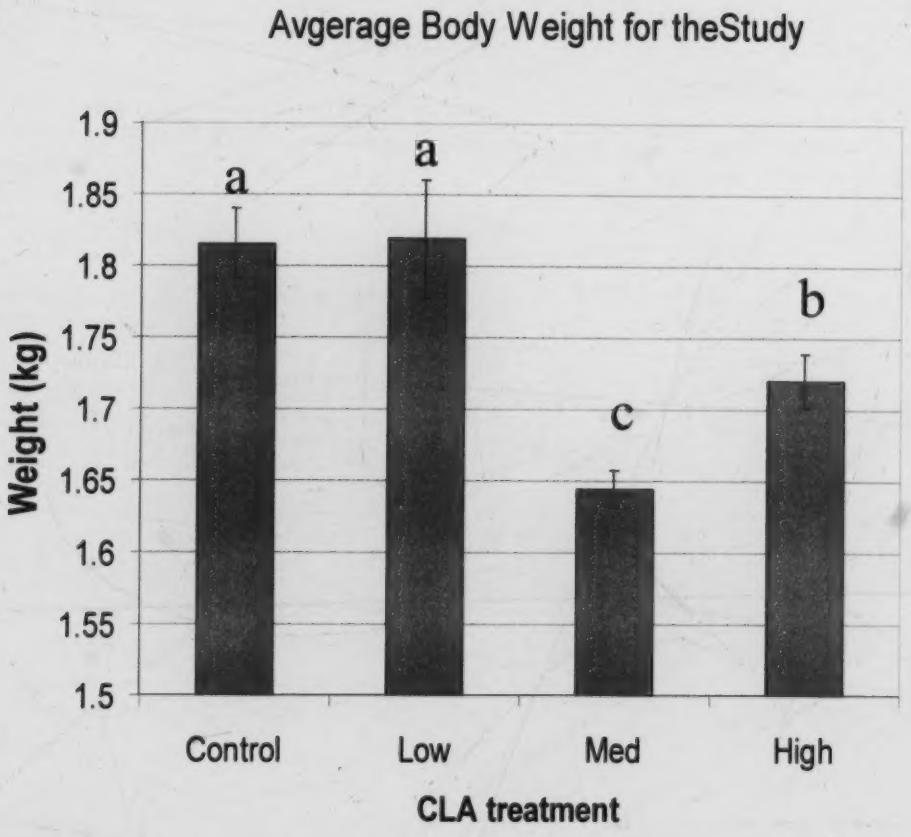
* above a column indicates a significant difference between isomers within a group (p,0.05)

Figure 2-4 Body Weight Changes



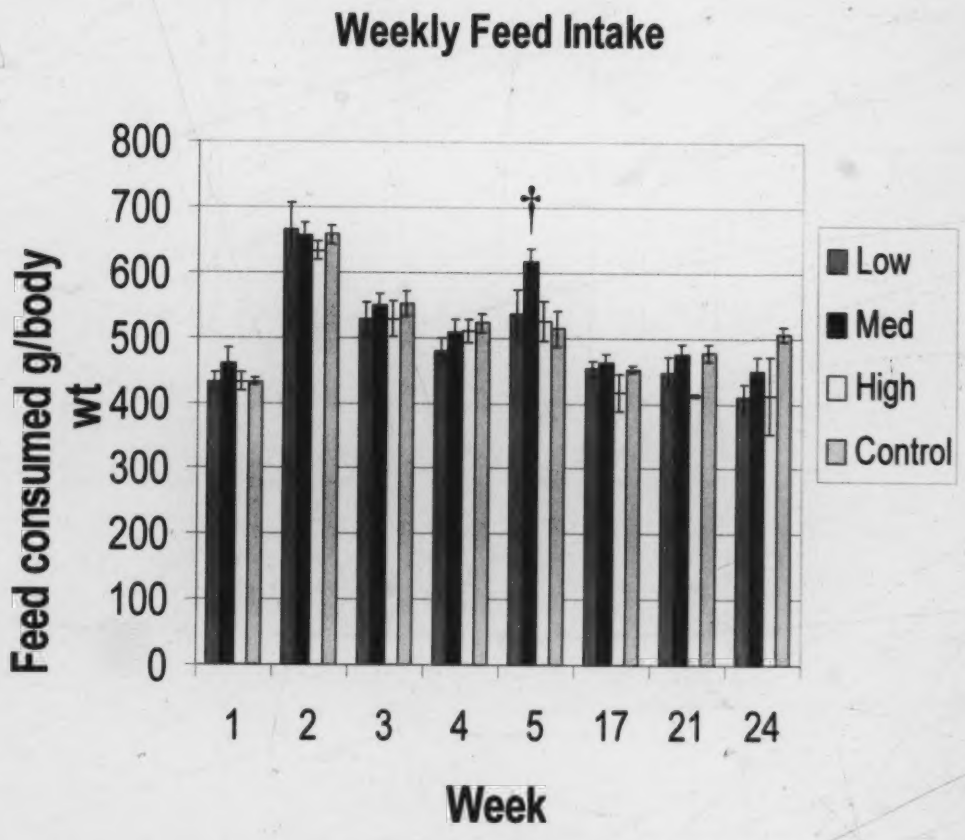
* indicates the point where group becomes and remains significantly lower than the low and control groups ($P<0.05$)

Figure 2-5 Body Weight Averages for Study



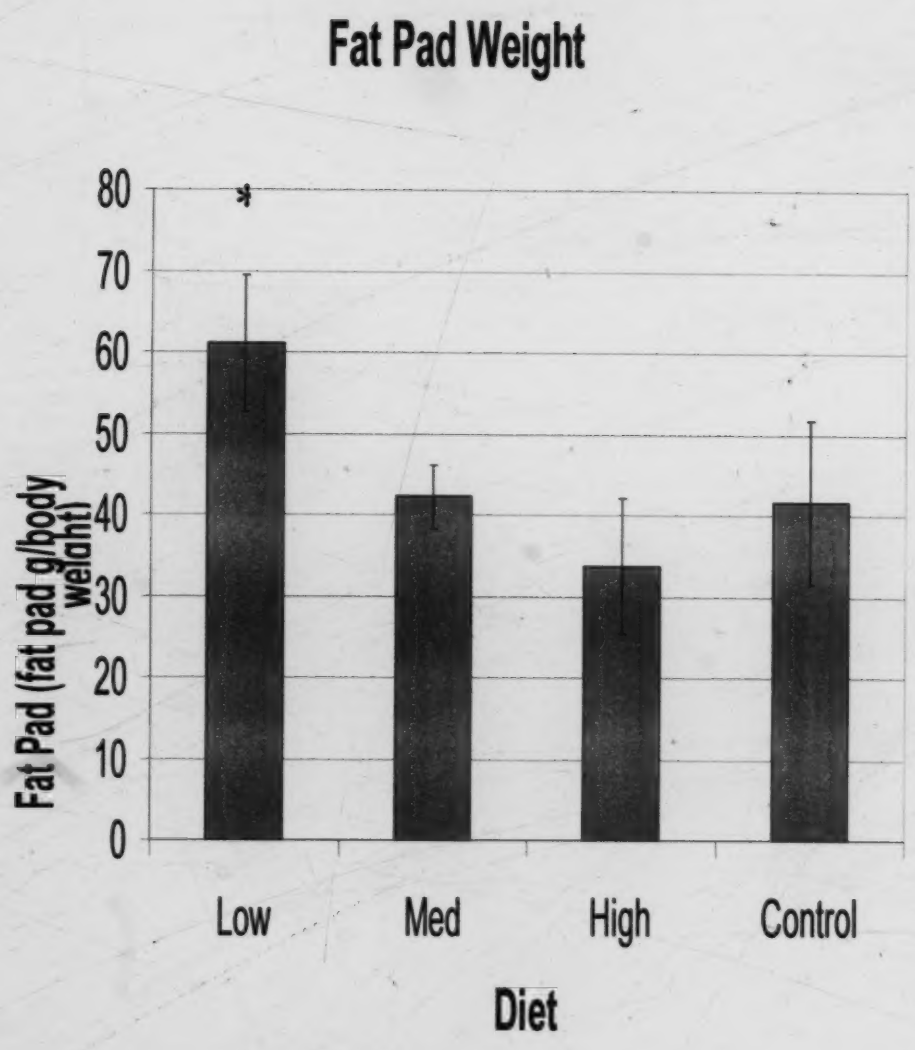
a,b,c above a column indicates significant differences between groups ($P<0.035$)

Figure 2-6 Feed Intake



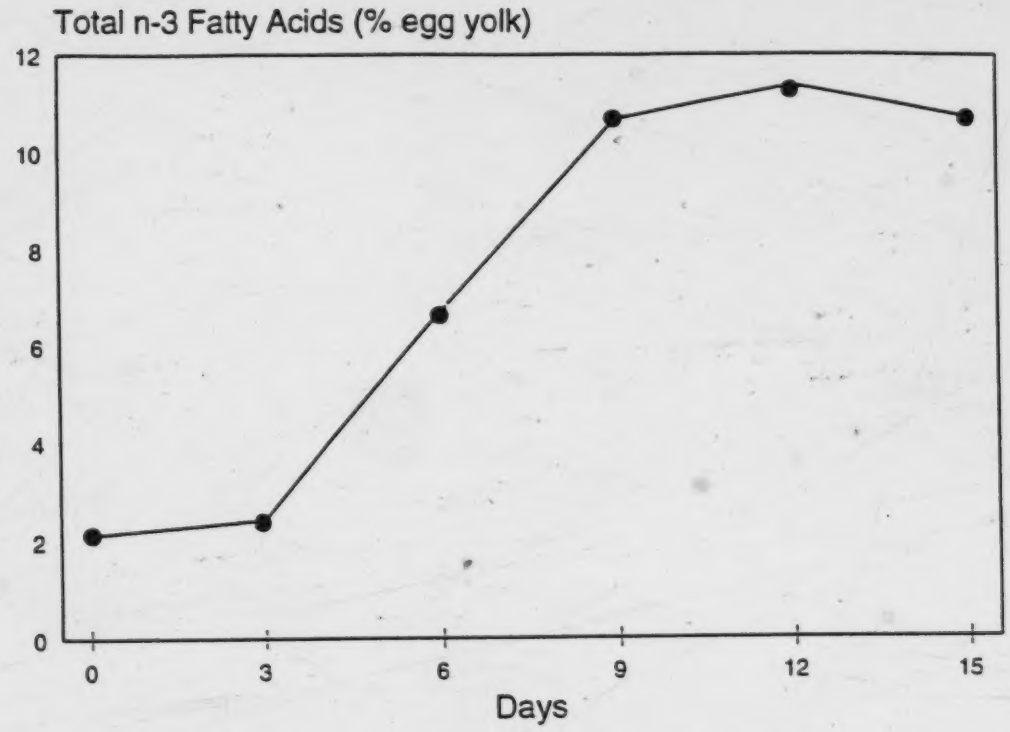
† Indicates a significant difference within a given week (P<0.007)

Figure 2-7 Fat Pad Weights



* indicates significance between groups (P<0.009)

Figure 2-8 Incorporation rate of n-3 fatty acids into yolk lipids



Accumulation of total n-3 fatty acids in the egg yolk of laying hens fed diets containing 3% α-linoleic acid from flaxseed (Sim et al. 1995)

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Chapter 3

Conclusion and General Discussion

A) Conclusion

CLA appears to play many different roles that may impact human health and nutrition. There are a variety of sources in which CLA can be obtained. Not only does CLA appear to have a place as an chemoprevention agent, but it has been linked to immune modulation, weight control, and decreased atherogenesis (Cook et al. 1993; Miller et al. 1994; Chin et al. 1994; Lee et al. 1994; DeVoney et al. 1997; Hayek et al. 1997). The potential implications of CLA for poultry and egg production are interesting. Although few clinical studies with CLA have been reported, this molecule has tremendous potential. This multipurpose molecule also appears to influence many different mechanisms, and many different species. It has been estimated from animal data, that humans would need to ingest approximately 3 g of CLA/day to see these health benefits (Ha et al. 1989). Presently, the consumption of CLA is much lower than the levels suggested to see an effect in humans, but it has been shown that even small dietary increases in CLA consumption sources can readily increase the level of CLA found in human plasma and tissue (Huang et al. 1994). This study reported that through a dietary regime in the laying hen, the yolk content of CLA in a single egg can be increased to levels similar to that of ruminant animal food products, or similar to that obtained in a glass of milk. Currently in the U.S is estimated on a per capita basis, that the average North American consumes about 234 eggs per year or 2/3 of an egg per day (Ensminger 1992). CLA enriched eggs may then be expected to contribute ~66mg of CLA/ week. While

this amount may not significantly increase the average consumption of CLA in a typical diet, it does present a start to engineering foods that contain higher levels of CLA.

B) General Discussion

With respect to the rate of incorporation of CLA into the yolk lipids, we found CLA followed a similar trend to the incorporation of n-3 fatty acids for the first 7-12 days (Sim et al. 1995). With n-3 fatty acids, the level of incorporation plateaus between day 9-12. We however observed a difference in the incorporation of CLA into the yolk lipids after 12 days. With CLA the level of incorporation appears to plateau around day 12, however days 24 and 36 were significantly higher than day 12 for both the medium and high CLA fed groups. The fact that CLA incorporation plateaus after 24 days for the medium group, and appears to continue to increase after 36 days in the high CLA fed group, suggests that CLA is preferentially incorporated in a dose dependent fashion. It is possible that CLA is favored when accumulated in the body fat stores of the hen, and deposited into the yolk lipids according to the level of body fat CLA stores. This would explain why the low group showed the first sign of a significant increase after 36 days, as well as why the medium group plateaued after 24 days. It may have taken 36 days for the low group to accumulate enough CLA to significantly raise the yolk CLA content, where as with the medium diet, the CLA stores of CLA may have reached an equilibrium between CLA from the feed and the CLA

incorporated into yolk lipids. Whatever the case, it appears that the group fed a high level of CLA may continue to increase the CLA content of the eggs after 36 days.

In terms of CLA effects on animal management, CLA was shown to correspond to previous literature regarding body weight and feed intake. The body composition analysis of this study lends supports to the body of evidence that CLA intake affects the ratios of fat mass to lean body mass. It was found that the high CLA group (1.0 g CLA/kg feed) had significantly less %body fat as compared to the low and medium CLA groups (0.01 g CLA/kg, 0.5 g CLA/kg). The control group had 3% more body fat than the high CLA group, but did not show significance. The reason for not finding significance from the control group could be the low *n*, or a problem factor that went undetected. The %water and %protein data verified the findings by Winchell et al. (Winchell 1998A) that the reduction in % body fat in rats, is replaced by an increase in % body water, rather than an increase in protein (lean body mass). The mechanism proposed for the observed reduction in body fat and the increase in lean body mass appears to be involved in reducing fat deposition, increasing lipolysis in adipocytes, and possibly enhancing fatty acid oxidation in adipocytes and muscle cells. (Pariza et al. 1997; Park et al. 1997) These observations, coupled with the fact that we found the occurrence of fatty livers in the control and low CLA fed group, suggest that CLA supplementation may be a beneficial preventative treatment for Fatty Liver Syndrome. A disease caused either by the excess

caloric intake, or by a deficiency in methionine or choline (Squires and Leeson 1988; Branton et al 1995). The effects of this condition are a result of the hepatocytes becoming distended with fat vacuoles, resulting in an enlarged fatty discolored liver that may rupture causing death (Rothenbacher et al. 1972; Squires and Leeson 1988; Branton et al 1995). Currently there is no method of preventing this disease, therefore CLA may be a means to reduce fat deposition, and control or reverse this disease.

There were no body weight differences between groups in any given week, except in week 5 where the medium group (0.5 g CLA/kg) appeared to eat more than the other groups. This could be in part due to the fact that in week five, the animals in the medium group were significantly smaller that the control and low CLA groups. Any food wastage that occurred in that week would be amplified when the food intake is presented in relation to body weight (feed * kg body weight $^{-1}$ /week). This difference in week 5 was not seen when comparing total feed/week. In first 4-6 weeks, there was a problem with the animals wasting food, thus giving an inaccurate account of their actual feed intake. This would explain why a difference when comparing average feed consumed per week was seen between weeks 1-5.

Increased feed efficiency has many positive implications for egg producers, such as possibly recouping the costs of supplementing diets with CLA through money saved from reduced feed costs. Another benefit may be the economic and

environmental impact resulting from decreased manure production and the associated processes of manure disposal (Alberta Agriculture 1995).

Feeding CLA to laying hens has the potential for benefiting the producer as well as the consumer population. In the present study, egg yolk lipids were enriched with physiologically active CLA to a level approaching that of ruminant animal food products. Currently there is no government or agency recommended level of CLA intake for humans due to the limited amount of research regarding human consumption and the health benefits of CLA. If further research establishes that CLA is as beneficial to humans as the animal studies currently show, increasing the consumption of CLA without increasing the amount of fat consumed in the diet will become a focus of interest. Thus, the engineering of foods to contain higher levels of CLA is one option to achieve this goal. Future research could examine whether feeding larger concentrations of CLA (1.0g CLA/kg feed and higher) will increase the incorporation of CLA into yolk lipids after 36 days and the length of time it takes to see a plateau in the rate of incorporation for these higher levels of intake. Another possibility for future research is the effect of CLA on flocks at high risk for fatty liver syndrome, and the likelihood of prevention and reversal of this disease.

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